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Research Article

Age-dependent variation of aedeagal morphology in *Agabus uliginosus* and the status of *A. lotti* (Coleoptera, Dytiscidae)

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Abstract

A doubt has arisen about the taxonomic status of *Agabus lotti* within the *Agabus uliginosus* species group due to morphological similarities and lack of molecular data. In this study, a comprehensive morphological and molecular analysis of specimens from Central Europe was conducted, focusing on the Hungarian population. Morphological comparisons of genital structures revealed age-dependent variations, suggesting a gradual transition from *A. lotti* to *A. uliginosus*. Molecular analysis of COI sequences further supported this hypothesis, showing minimal genetic differences among most specimens, with only one individual exhibiting distinctiveness. Therefore, *A. lotti* **syn. nov.** must be regarded as a junior synonym of *A. uliginosus*. Our findings also highlight the need for additional multi-marker studies covering a broader geographic range and including both molecular and morphological approaches to elucidate the taxonomic and phylogenetic relationships within this species group. The inclusion of Hungarian samples notably enriched the diversity of haplotypes, emphasizing the importance of expanding sampling efforts in future research.

Key words: Aedeagal variability, age determination, COI, DNA barcoding, internal sexual organs, phallobase

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Introduction

The *uliginosus* group, within the genus *Agabus* (Dytiscidae, Agabinae), as delineated in its present form by Larson (1989) and Nilsson (2000), and currently summarized by Nilsson and Hájek (2024), comprises seven species: *A. amnicola* (J. Sahlberg, 1880) with a Holarctic distribution, *A. jacobsoni* Zaitzev, 1905, *A. lotti* Turner, Toledo & Mazzoldi, 2015, *A. uliginosus* (Linnaeus, 1761), *A. uralensis* Nilsson & Petrov, 2006, and *A. vereschaginae* Angus, 1984 with a distribution in the Palaearctic region, while *A. margareti* Larson, 1975 (= *A. margaretae* Larson, Alarie & Roughley, 2000, as unjustified emendation of *margareti*; see also Fery and Bouzid 2016) is a Nearctic species. Previously, Larson (1989), Nilsson and Holmen (1995), and Nilsson and Toledo (1999) grouped *A. amnicola*, *A. jacobsoni*, *A. uliginosus*, *A. vereschaginae*, *A. margareti*, and *A. falli*

(Zimmermann, 1934) within the uliginosus group; however, Nilsson (2000) later excluded *A. falli* from this group and transferred it into its own group. Subsequently, Nilsson and Petrov (2006) distinguished the Asian *A. uralensis* from *A. uliginosus* based on male genitalia, thereby elucidating the distribution of *A. uliginosus* in the western Palaearctic. The description of *A. lotti* as the sixth Palaearctic species within the *uliginosus* group took place in 2015 (Turner et al. 2015). This species, endemic to Central Europe, can be readily distinguished from *A. uliginosus* by its small phallobase and thin bases of parameres, in contrast to the considerably enlarged phallobase and thickened, more sclerotized bases of adhering parameres in *A. uliginosus*. As described by Turner et al. (2015), "penis in lateral view basally sinuate expanding medially, followed by a sinuate contraction toward the narrowing apex; the external margin progresses evenly in a gradual inward curve until the three quarters to the apex where a straight section completes the apex". Among the external characters, the authors highlight the more elongated body shape of *A. uliginosus*, compared to the laterally more expanded body shape of *A. lotti*.

According to Lawrence et al. (2010), a coleopteran aedeagus comprises the phallobase, parameres, and penis. In the trilobate type aedeagi, the parameres are paired structures articulated with the posterior end of the phallobase, which is the basal portion of the aedeagus. Lawrence et al. (2010) notes that in many Coleoptera (including Adephaga), the phallobase appears to be absent, having become membranous or fused to the parameres. Additionally, they define the tegmen as a slender sclerite articulated with sternite IX or with the connecting membrane between sternite IX and the phallobase. These structures were consequently illustrated by Franciscolo (1979) for European species. Miller and Bergsten (2016) described various types of male genitalia within Dytiscidae and denoted that the median lobe may be either bilaterally symmetrical (Dytiscinae, most Hydroporinae) or asymmetrical and variously twisted.

In insects, particularly adephagous beetles, it is clearly shown that internal reproductive organs develop gradually and significantly increase in size with age, progressing from immature to teneral adults and eventually to fully sclerotized, old individuals. Various age classes were therefore described for both females and males (Dettner et al. 1986). While degeneration of internal genitalia in elder beetles typically occurs post-reproduction, instances of genital reactivation have been observed in beetles living for extended periods, sometimes several years (Blunck 1912). Fresh weights, though subject to fluctuations due to varying food intake, can serve as an approximate indicator of age, owing to the gradual increase in biomass of ectadenies (= accessory glands), testes, and ducti ejaculatori in males, and ovaries, eggs, oviducts, and vagina in females (Dettner et al. 1986). The species within the *A. uliginosus* group in some way differ compared to most other Dytiscidae: the shape of the male genitalia continues to develop after the adult beetle has emerged, as first noted by Nilsson and Petrov (2006).

DNA barcoding offers an effective method for species-level identification and mapping genetic diversity, enabling the detection of atypical specimens for thorough taxonomic analyses and as such, give much potential to reveal new species or to review the taxonomic status of previously described taxa (Hajibabaei et al. 2007). Among the genetic markers used in DNA barcoding, mitochondrial cytochrome c oxidase subunit I (COI) has proven to be particularly

effective for species identification across the animal kingdom, with its divergences facilitating discrimination even among closely related species, especially within Arthropoda (Hebert et al. 2003a, 2003b).

Despite the potential of DNA barcoding, COI-based DNA reference databases remain incomplete (e.g., Weigand et al. 2019), including for adephagous aquatic beetles (Csabai et al. 2023). Currently, publicly available sequences exist for only two of the seven species within the *uliginosus* group in databases such as BOLD (Ratnasingham and Hebert 2007) and GenBank (Benson et al. 2013). There are 17 public/published sequences from Germany and Finland for *A. uliginosus*, all of which are classified within a single BIN (Barcode Index Number, Ratnasingham and Hebert 2013), with an average distance of 0.38% and a maximum distance of 1.52%. Additionally, a single sequence from Canada is available for *A. margareti*, forming a separate BIN (BOLD:ACA6100), with a 6.9% distance from the nearest neighbor, the *A. uliginosus* BIN (BOLD:AAY8849). Venables (2016), in her PhD thesis on the molecular systematics of the Agabini, included *Agabus uliginosus* and *A. lotti* in some phylogenetic analyses, albeit with only single specimens analyzed. These two species, with *A. lineatus* (*lineatus* group), represented a monophylum, with *A. lotti* and *A. uliginosus* positioned very closely.

Given the morphological similarity and genetic proximity of these species, it is more appropriate to reach a consensus based on various methods rather than relying solely on a single approach. Therefore, in this study, alongside describing age-dependent variation in aedeagal morphology in *A. uliginosus*, we adopt an integrative approach, evaluating internal morphological features, developmental characteristics, and COI-sequences to revisit the taxonomic relationship and status of *A. uliginosus* and *A. lotti*.

Materials and methods

Morphological studies

As the initial step of the morphological analyses, we examined pinned dry specimens and ethanol-preserved specimens obtained from previous studies, from various regions including Germany (Baden-Württemberg, Bavaria, Rhineland-Palatinate, Saxony, Schleswig-Holstein), Austria, Hungary, and Southern Poland. The material was sourced from collections maintained by KD (Bayreuth, Germany), the Senckenberg Deutsches Entomologisches Institut (Müncheberg, Germany), and the Department of Hydrobiology at the University of Pécs (Pécs, Hungary).

Male specimens, regardless of their previous identifications as *A. lotti* or *A. uliginosus* [hereinafter *A. uliginosus* s. l. (sensu lato)], were examined for their primary external sexual organs. In cases of 29 male specimens, internal male sexual organs were dissected and analyzed in detail. Careful dissection was necessary to obtain intact phallobases, often involving the removal of tightly adhering basal parts of parameres, which were prone to damage during dissection. Age determination of freshly alcohol-preserved specimens from Hungary was conducted. The body weights of ethanol-preserved specimens from Hungary were measured one hour after deposition on filter paper using a Professional Digital Mini Scale, TL-series (1 mg–20 g; Shenzhen Union Technology, China). Additionally, a few data on ectadenia were recorded from freshly collected specimens from Bavaria.

In the next step, specimens were captured and kept alive from saline habitats of the Kardoskúti-puszta plain in southeastern Hungary (42 specimens) on May 1, 2023. The living Hungarian specimens were immediately sent to Germany by express mail using fishing bait boxes filled with heavily moistened filter papers. After arrival, the specimens were frozen, and fresh weights were measured 30 min after defrosting. Sixteen male specimens were selected for detailed morphological analyses. Soft translucent chitinous structures at phallobases were dyed darkened using a 1% aqueous or 1% ethanolic solution of pyrogallol (Seifert 1995) for microscopic investigation. Darkening was controlled after 1 h to 2 days to achieve best results.

To evaluate aedeagal morphology, measurements were taken on all available individuals, including the length of the penis from apex to distal corner of the basal part (a on Fig. 1A), the lengths of the penis base from the distal corner of the basal part to the basal part defined by the imaginary extension of the outer curvature of the penis on a line defined by a (b on Fig. 1A), the length of the sclerotized phallobase from the imaginary extension of the outer curvature of the penis to the basal sclerotized base of the phallobase in line with a + b (c on Fig. 1A), and the thickness of the non-sclerotized soft and translucent membrane of the phallobase (d on Fig. 1A). Basal pieces of both parameres and tegmina were also observed. Of the 45 male specimens of *A. uliginosus* s. l. examined in detail, fresh weight data were measured for 26 individuals (10 ethanol-preserved and 16 frozen-preserved, all from the Hungarian populations), while the most critical morphological parameters of the genitalia were recorded in 44 cases. All the beetles and genitalia were examined, dissected, and measured using a ZEISS binocular and a ZEISS microscope (Standard 16). Phase-contrast photographs were captured using an OLYMPUS BH2-RFCA microscope. All morphometric measurements and visual inspection results were compiled in Suppl. material 1: table S1.

Phenological records

To gain insight into the temporal distribution of records for both species, we conducted a comprehensive search for records with at least the month of collection. We utilized a variety of sources, including academic papers, specifically for *A. lotti* records: Turner et al. (2015), Boda et al. (2019, 2023), and Rocci et al. (2021). We accessed data from the GBIF database (for specific dataset citations see Suppl. material 1: table S2) and from the verified public database of "Izeltlabuak.hu" citizen science initiative (http://www.izeltlabuak.hu) for both species, and obtained collection records from the collection of KD (Bayreuth, Germany), the Senckenberg Deutsches Entomologisches Institut (Müncheberg, Germany), and the Collection of the Department of Hydrobiology of the University of Pécs (**DHUP**) for unpublished data on both species (Suppl. material 1: table S1).

Molecular studies

DNA barcode amplification and sequencing

In cases of the 16 Hungarian male specimens that were maintained alive, later frozen, weighed, and subjected to morphological analysis, we removed one hind leg from each specimen and preserved them in 96% ethanol, thus enabling

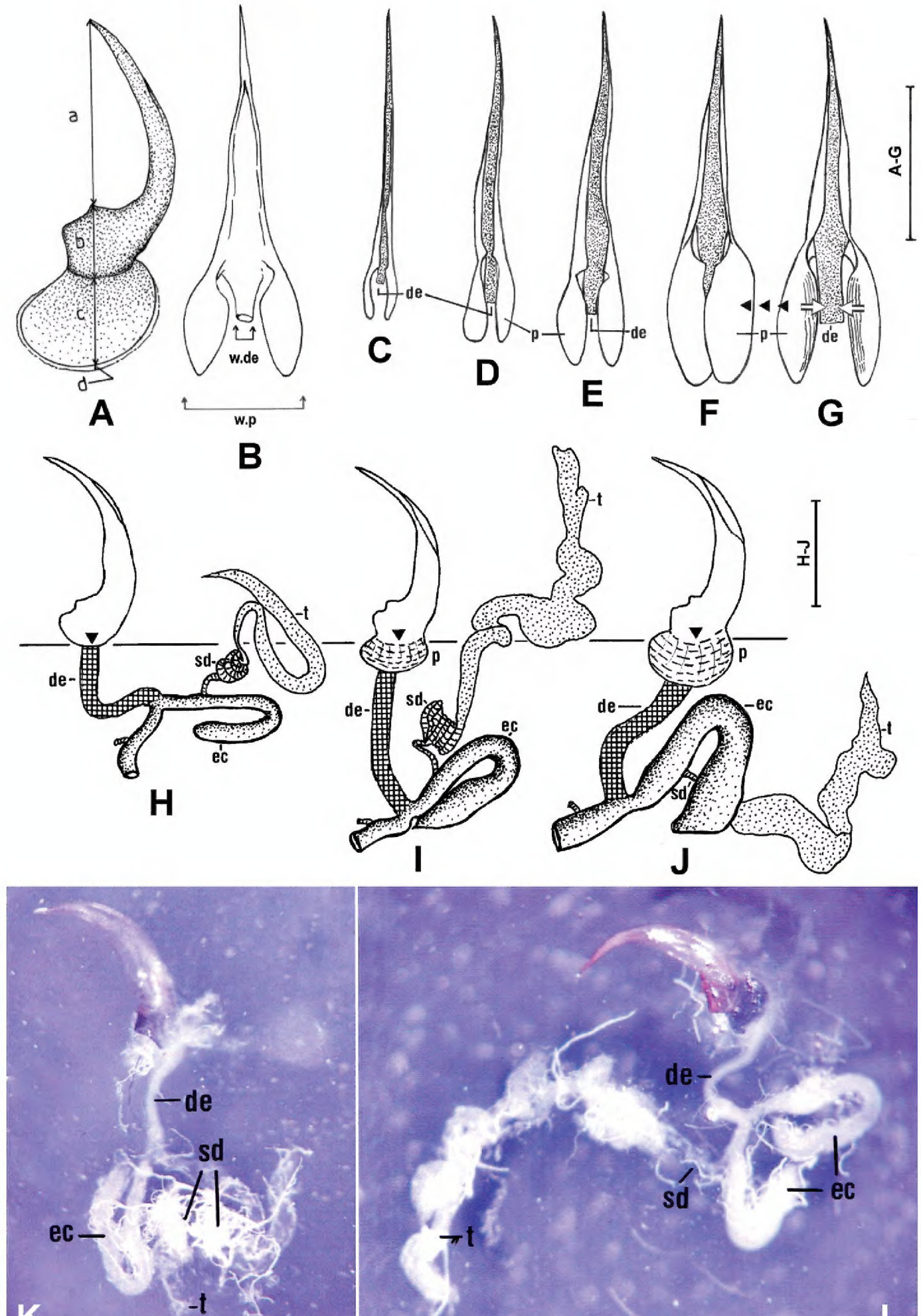

Figure 1. Genital morphology and its changes from young and elder specimens of *Agabus uliginosus* sensu lato **A, B** morphology and aedeagus of an elder specimen in right side lateral (**A**) and dorsal (**B**) views, with measured parameters a-d. **C–G** Dorsal views of aedeagi from different age classes of *A. uliginosus* s. l. **C** corresponds to *A. lotti*, while **D–G** to *A. uliginosus* s. str. with increasing age **C–E** Hungarian population **F, G** old male specimen from Rudna, Mühlgast, Poland **F, G** show the same aedeagus isolated from a specimen preserved in ethanol, then dried for 5 min (**F**), and for 15 min or more (**G**). Black arrows on G symbolize movements of phallobases **H–L** side view of aedeagi and morphology of internal genital organs of an immature (**H, K,** #H04), an elder (I, #H27) and an old (**J, L,** #H30) specimen of *A. uliginosus* s. l. from the Hungarian population. H and K correspond to *A. lotti*, J and L to *A. uliginosus* s. str, while I shows a young specimen of *A. uliginosus* s. str. as a transition between them. Abbreviations: de: ductus ejaculatorius (checkered in **H–J**), ec: ectadenies (dotted in **H–J**), sd: spermatic duct (cross-striped in **H–J**), p: phallobase, t: testes (dotted in **H–J**), w.p: width of phallobase, w.de: width of ductus ejaculatorius. The triangles and the interrupted horizontal line in **H–J** illustrate the origin of the phallobase as indicated by b in A. In K (#H04) and L (#H30) anterior margin and origin of ductus ejaculatorius is marked by arrows. Scale bars: 1 mm (different scales refer to **A–G** and **H–J**). For **K** and **L**, 20-fold magnification was used.

us to conduct molecular studies (DNA barcode analysis). Samples were processed for sequencing at the Department of the Invertebrate Zoology and Hydrobiology, University of Lodz, Poland, by ZK and TR. DNA was extracted from a leg segment of the specimens using the Chelex procedure (Casquet et al. 2012). A 650 bp fragment of COI was amplified using primers LCO1490-JJ and HCO2198-JJ (Astrin and Stüben 2008), under the following PCR conditions: initial denaturing for 60 s at 94 °C, followed by five cycles of 30 s at 94 °C, 90 s at 45 °C, 60 s at 72 °C, 35 cycles of 30 s at 94 °C, 90 s at 51 °C, 60 s at 72 °C, with a final 5 min extension at 72 °C (Hou et al. 2007). PCR was conducted in 12 µL volumes, containing 1.1 µL of DNA template, 1 µL of each primer, 6 µL of DreamTaq PCR Master Mix (Thermo Scientific) and 2.9 µL of nuclease-free water. Then 2 µL of each reaction product was checked with 1% agarose gel electrophoresis to confirm amplification. 5 µL of PCR products were purified with Exonuclease I (2 U, EURx) and alkaline phosphatase Fast Polar-BAP (1 U, EURx), according to the manufacturer's instructions. One-way Sanger sequencing was outsourced to Macrogen Europe (Amsterdam, the Netherlands). The sequences were edited, trimmed of primers, and aligned using Geneious 11.1.5 software package (Kearse et al. 2012).

DNA data assembly and barcode analyses

We used the BLAST (Altschul et al. 1990) searches to identify all the new sequences to confirm their identity based on already published sequences. All obtained COI sequences were deposited in GenBank (PP464831–PP464847; Suppl. material 1: table S3). Additionally, the DNA sequences were compared with 17 publicly available *A. uliginosus* s. l. and one *A. margareti* sequences retrieved from the public repository (Pentinsaari et al. 2014; Hendrich et al. 2015; Rulik et al. 2017; Linard et al. 2018) of the Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert 2007), and altogether deposited in a separate DS-AGHUZSK dataset, where all the relevant metadata and sequence trace files are publicly available (http://dx.doi.org/10.5883/DS-AGHUZSK). Intra- and interspecific genetic distances were calculated based on the Kimura 2-parameter model (K2P; Kimura 1980), using the analytical tools of the BOLD workbench (Distance Summary, and Cluster Sequences). A phenogram (see Suppl. material 1: table S3 for details) was constructed in MEGA X (Kumar et al. 2018) with the neighbor-joining method (Saitou and Nei 1987), based on the K2P distance (Kimura 1980), with a bootstrap test (1000 replicates). We estimated the genetic diversity, i.e., the haplotype diversity (h) and nucleotide diversity (π) (Nei 1987), using the DnaSP v. 5 software (Librado and Rozas 2009). The relationships within *A. uliginosus* were displayed through a median-joining network using PopART (Leigh and Bryant 2015).

Species delimitation methods

We used three distinct species delimitation methods harboring two different approaches. Initially, we used two distance-based methods. First, we employed BINs (Ratnasingham and Hebert 2013) integrated into BOLD, wherein COI DNA sequences, whether newly submitted or pre-existing, are organized and grouped into separate clusters according to their genetic distances. Secondly,

we used the ASAP procedure, developed by Puillandre et al. (2021), which is a hierarchical clustering algorithm designed explicitly for species partitioning, leveraging pairwise distance distribution. The ASAP analysis was run with the iTaxoTools v. 0.1 software (Vences et al. 2021). We applied the Bayesian implementation of the multi-rate PTP (mPTP -https://mcmc-mptp.h-its.org/mcmc/; Kapli et al. 2017) for the phylogeny-based delimitation method. This method incorporates MCMC sampling, offering a rapid and comprehensive evaluation of the deduced delimitation. We used Maximum Likelihood (ML) trees calculated with MEGA X (Kumar et al. 2018) as input for analysis.

Results

Morphological studies

Among the 45 male specimens of *Agabus uliginosus* s. l. subjected to detailed examination, a range of developmental stages were observed, including freshly hatched individuals with soft cuticles, very young, young, and more mature specimens (Suppl. material 1: table S1). Analysis of the genitalia revealed a correlation between age and the increasing lengths of phallobases (p), testes (t), and ectadenies (ec), which became progressively longer and thicker (Fig. 1H–J). The measurements (b+c) were found to be positively correlated with the lengths of ectadenies (Fig. 2B) and the fresh weights of the specimens (Fig. 2A), both in ethanol-preserved and frozen material, indicating that fresh weight roughly reflects the gradual enlargement of the phallobase. Additionally, the widths of phallobases in dorsal view (see Fig. 1C–G) significantly increased with age, with the widths of ejaculatory ducts (Fig. 2C). Moreover, total lengths of aedeagi also showed an increase (Fig. 1C–J). This significant swelling process (Fig. 1C–G), observed from young to elder specimens, is noteworthy because it is unusual among adephagous beetles, as diameters in dorsal views of phallobases and whole aedeagi typically represent species-specific values. Consequently, changes in diameters of aedeagi in dorsal views, as well as varying shapes of the entire aedeagi ranging from straight to curved and even asymmetrical organs, were observed (Fig. 1C–G). Furthermore, an old, freshly dissected specimen (Fig. 1F) exhibited a different shape compared to the same specimen after drying (Fig. 1G).

Significant correlations were seen in both types when comparing body length and width (Fig. 2D). However, the *A. uliginosus* s. str. specimens showed higher and more variable body lengths, with relatively constant widths. Conversely, in *A. lotti* type, both body length and width showed variability. We did not see significant differences in body ratio (length/width) between *A. uliginosus* s. str. (mean = 1.75, n = 11) and *A. lotti* types (mean = 1.77, n = 9) (Mann-Whitney U test, U = 36.5, z = 0.95, p = 0.34).

Another distinctive characteristic observed in male representatives of the *A. uliginosus* group is the soft and translucent external border of the phallobase (d in Fig. 1A), which is not sclerotized (Fig. 3A–C; arrows). Under higher magnification (Fig. 3C), radially arranged filaments were visible, indicating potential ongoing sclerotization processes along these structures. Elder males, as shown in Fig. 3D, showed nearly complete sclerotization of section d, which is greatly reduced. Additionally, the sclerotized phallobase displayed both radial

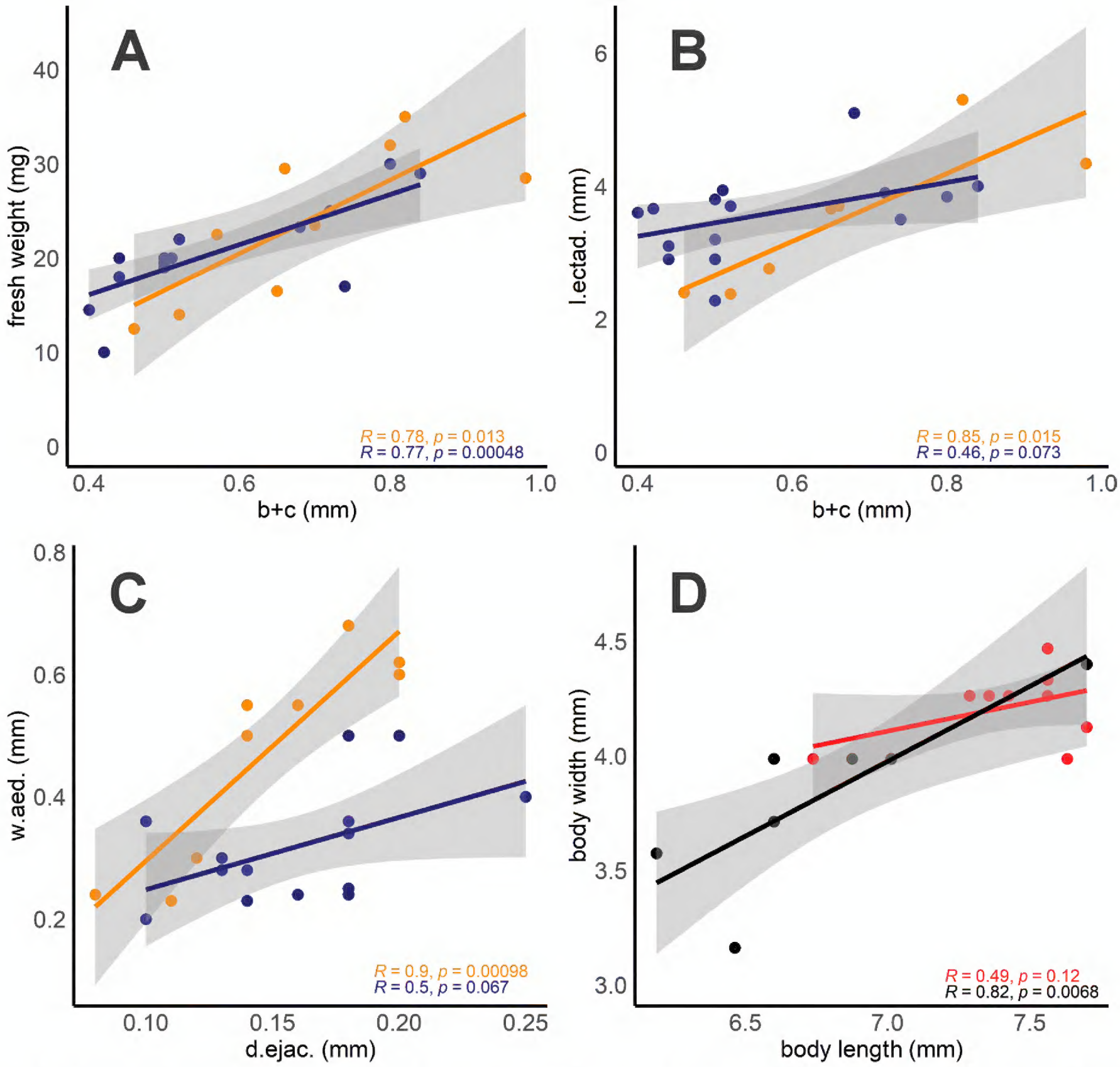

Figure 2. A Fresh weights and **B** length of accessory glands (= ectadenies, l.ectad. here and ec in Fig. 1H–J) as function of the size of the phallobase (b+c here and in Fig. 1A) **C** widths of the bases of the aedeagi in dorsal view (w.aed.) compared with diameters of ejaculatory ducts (d.ejac.) **A–C** measurements were made in ethanol-preserved (orange) and frozen (blue) male specimens of *A. uliginosus* sensu lato from Hungary **D** comparison of the body lengths and widths of the males showing the differences and the variation in body shapes in *A. uliginosus* sensu stricto (red) and *A. lotti* (black) types.

and circular filaments (Fig. 3D). The widths of these non-sclerotized phallobase borders (d) in elder and old male specimens varied distinctly. This unique morphological detail, optimally visible after artificial darkening with reagents, may suggest an extension of the phallobase even in old males, potentially indicating multiple gonadal activities during several months or even years. Consistent with these findings, both basal parts of parameres and tegmina broadened (tegmina also lengthened) and became increasingly sclerotized with age from youngest to the oldest, as shown on Fig. 4A–D, respectively.

To sum up, during imaginal life, respective imaginal age classes of *A. uliginosus* s. l., the sclerotization of area c gradually increased, phallobases enlarged, parameres and tegmina lengthened, foreclaws became well-wore from immature specimens (see Figs 1H, 3A–C, 4A, B) to elder and highly aged adults (Figs 1I, J, 3D, 4C, D). Hereby, lengths below 0.6 mm (b + c) in immature

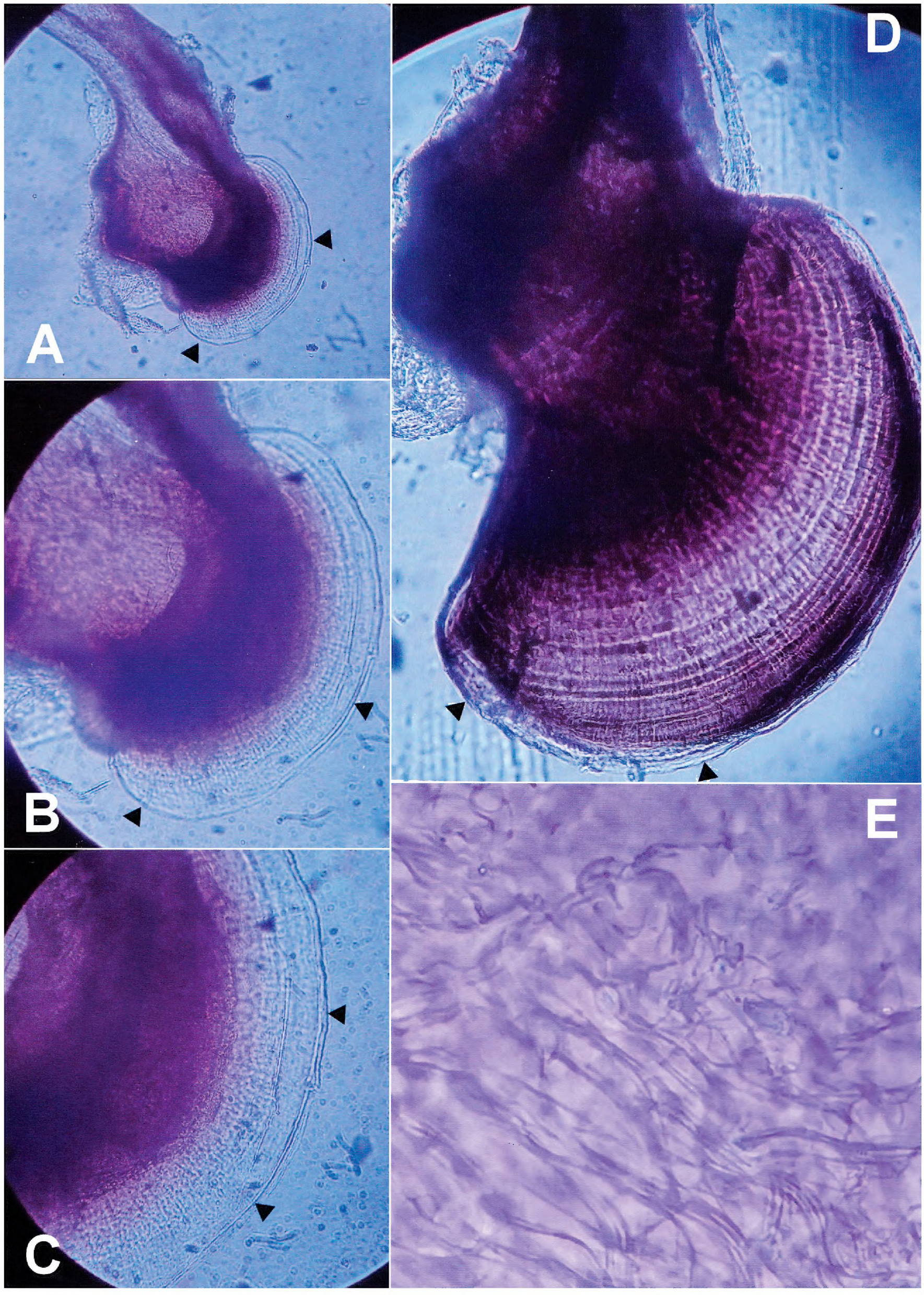

Figure 3. Translucent peripheral area of phallobase (d in Fig. 1A) of male *A. uliginosus* s. l. **A–C** #G06 specimen from Crailsheim (Germany) **D** nearly completely sclerotized basal area of aedeagus of an old male *A. uliginosus* sensu stricto #P01 specimen from Rudna, Mühlgast, Poland with both radial and circular filaments. Arrows indicate the outer margin of d in Fig. 1A, and radially arranged filaments between c and d are clearly visible, especially on C **E** spermatozoans from spermatic duct of elder male #H27 from Hungary (see also Figs 1I, 4C). Magnifications: **A** 32-fold **B** 100-fold **C** 200-fold **D** 100-fold **E** 400-fold.

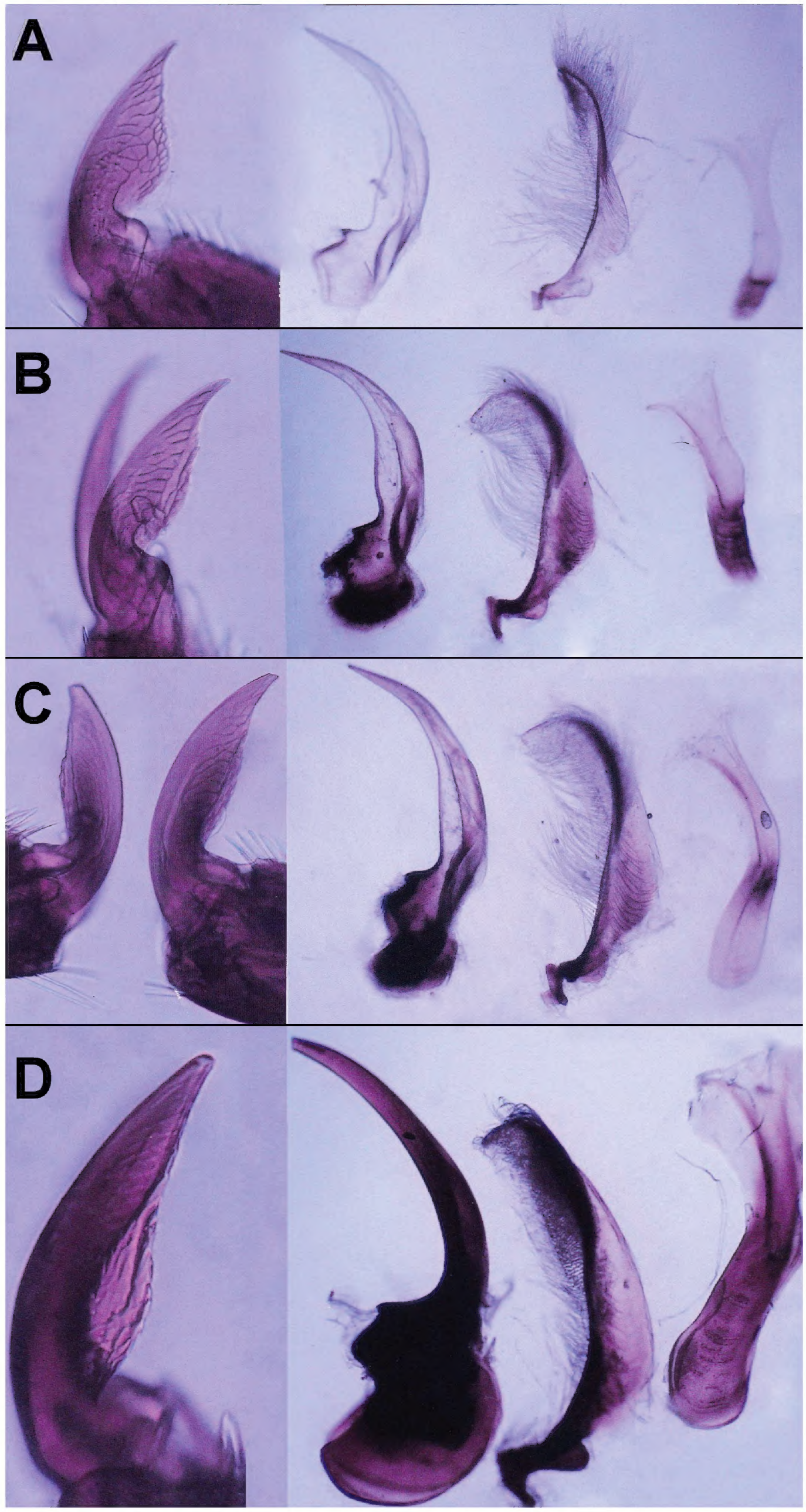

Figure 4. Morphological characteristics (from left to right in all panels: foreclaws, aedeagi, parameres, tegmina) in *Agabus uliginosus* s. l. males of different age categories **A** #H19 **B** #H11 **C** #H27 specimens, all from Kardoskút, Hungary **D** #G02 from Salemer Moor, Ratzeburg, Germany. **A** represent *A. lotti* type **B–D** represent *A. uliginosus* s. str. type.

specimens were tentatively associated with *A. lotti* (e.g., Fig. 1H, K). Conversely, males with b+c greater than 0.6 mm are interpreted as *A. uliginosus* s. str. (see Figs 1J, L, 3). Based on genital morphology and size proportions, approximately half of the examined males (20 specimens) showed characteristics typical of *A. uliginosus* s. str. type, 21 specimens were of *A. lotti* type, while three individuals showed completely transitional characteristics and values.

Phenology records

Altogether, we gathered more than 1600 dated records for *A. uliginosus* s. l. and 105 for *A. lotti* specifically. The majority of records for both species are concentrated in the months from March to June, with a notable peak in May. However, while data for *A. uliginosus* s. l. are spread across all seasons, with more than 300 records from August to February, records for *A. lotti* are limited to the period from March to July (Suppl. material 1: table S4). Notably, only two records for *A. lotti* are available in July, one from Austria and one from Slovakia, likely originating from higher elevation sites.

Molecular analyses

We successfully sequenced 16 specimens of *A. uliginosus* s. l. from Hungary. Comparison with all available *A. uliginosus* s. l. sequences in BOLD, revealed a relative homogeneity of the sequences regardless of whether our newly sequenced specimens morphologically classified to the *A. uliginosus* s. str. or *A. lotti* type. The Neighbor-Joining tree (Fig. 5) and Median-Joining network (MJ network) (Fig. 6) clearly demonstrate that the newly sequenced individuals classified as '*A. lotti*' or *A. uliginosus* s. str are not distinct from each other or from the publicly available sequences in the BOLD database. While more '*A. lotti*' sequences are located on the side branches of the MJ network, the two types remain intermixed throughout the entire network, with their haplotypes showing complete overlap (Fig. 6B).

In our sample from a single Hungarian population, we identified 12 haplotypes, with an additional seven haplotypes from public sequences in BOLD, resulting in a total of 19 known haplotypes and two intermediate haplotypes in the entire network (Fig. 6). From Germany and Finland, seven and two haplotypes, respectively, were identified. The central haplotype, comprising ten sequences, includes individuals from all three countries (Fig. 6A) and both types (Fig. 6B), i.e., the Hungarian individuals classified as both *'A. lotti'* and *A. uliginosus* s. str., sequences that are 100% identical to each other and to sequences from Germany and Finland. Apart from the central haplotype, two haplotypes are represented by two copies, while the others are represented by only one.

Species delimitation methods yield consistent classifications for most sequences, with only a discrepancy observed in the case of three Hungarian sequences classified as 'lotti' type and one as uliginosus s. str type. The most conservative method (ASAP) assigns all sequences to a single Molecular Operational Taxonomic Unit (MOTU). For delimitation based on BINs, all but one new sequence aligned to the BIN BOLD:AAY8849, which already contained all 17 previously known *A. uliginosus* s. l. sequences. Within this relatively compact

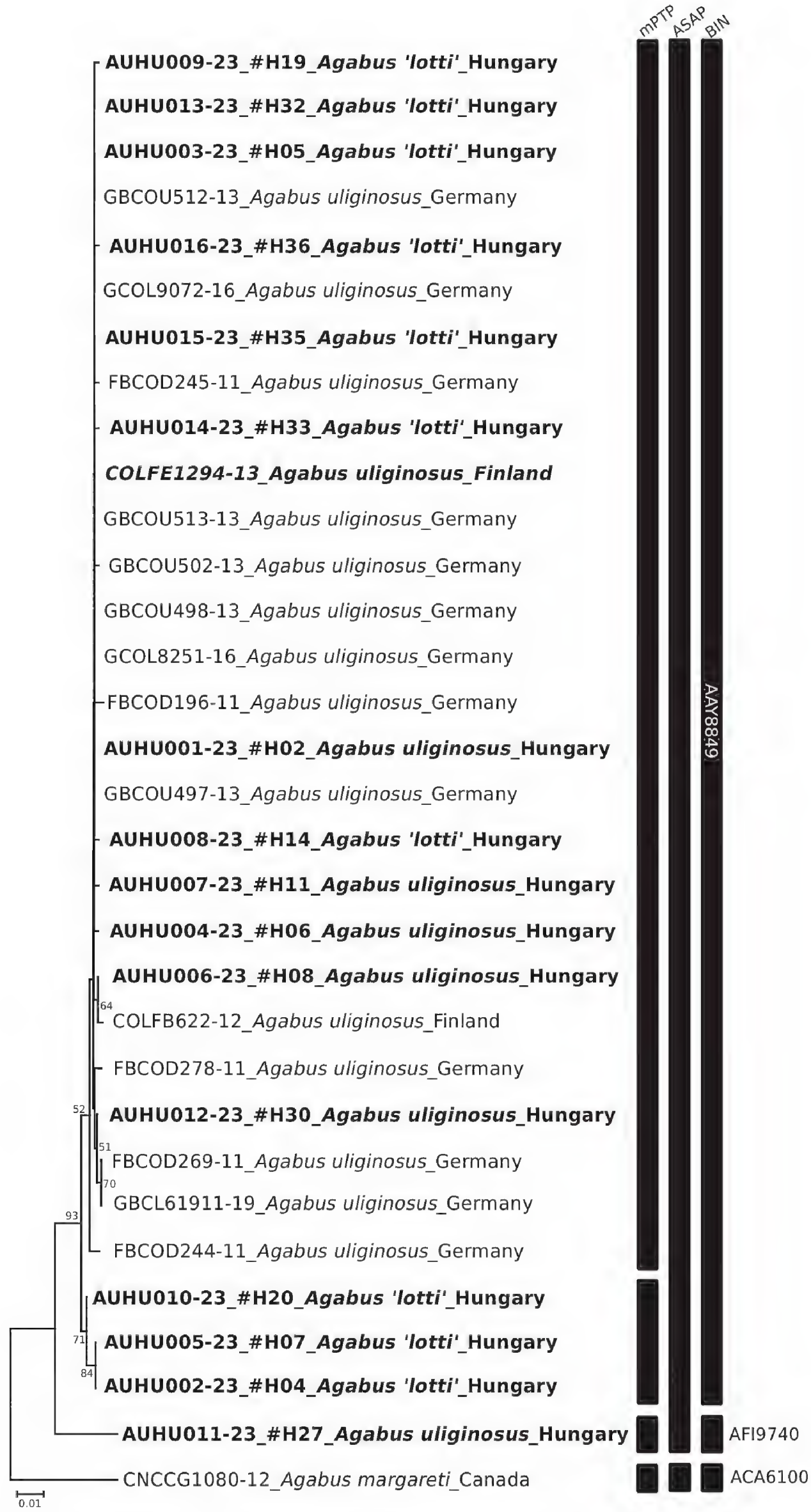

Figure 5. Neighbor-Joining tree based on COI K2P pairwise distances for sequences of *Agabus uliginosus* s. l. and *Agabus margareti* (BOLD taxonomic backbone uses the incorrect name *A. margaretae* for this species, see introduction for more information) used as an outgroup. Names written in bold have genitalia examined for morphological discrimination between *'lotti'* and '*uliginosus* s. str.' types. Each sequence name consists of parts separated by a lower dash: (1) BOLD Process ID, and for the Hungarian specimens the specimen codes used in morphological analyses are also given, (2) species (types), and (3) country of origin. The results of the three species delimitation methods are indicated by vertical bars (BIN numbers given). Only bootstrap supports higher than 50% are shown.

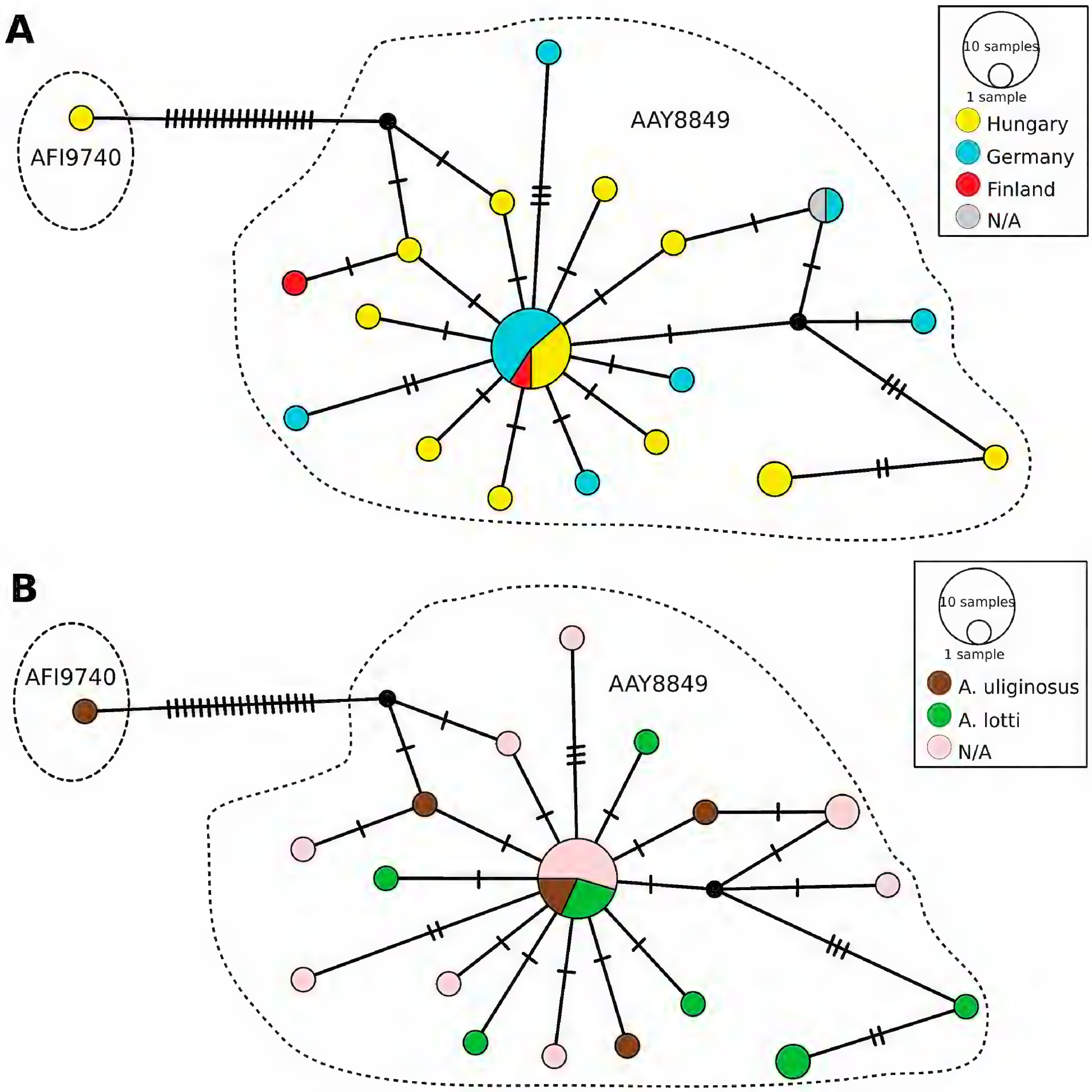

Figure 6. Median-Joining network showing the relationships among the haplotypes of *Agabus uliginosus* s. l. in the context of **A** country of origin **B** morphotypes. Colors indicate individuals representing different countries of origin or morphological types. Each bar represents one substitution, whereas small black dots indicate undetected/extinct intermediate haplotype states. The sizes of the circles are proportional to the frequencies of haplotypes. Dashed lines encircle separate BINs.

and homogeneous BIN, the average distance was 0.38%, and the maximum distance was 1.52%. However, the specimen #H27 was grouped separately and formed a singleton BIN (BOLD:AFI9740), which became the nearest neighbor of the *A. uliginosus* BIN, with a distance of 3.7%. In comparison, the only species of the species group whose sequence was available in the BOLD (*A. margareti*, BOLD:ACA6100 singleton BIN) showed a distance of 6.9% to its nearest neighbor, which, like that of the new #H27 BIN, was also the 'original' *A. uliginosus* BIN. Adding specimen #27, so including both *A. uliginosus* BINs, the average within-species distance increased to 0.72%, while the maximum within-species distance was 4.57% (Suppl. material 1: table S5.).

Discussion

Morphological characterization of *A. uliginosus* sensu lato, including *A. lotti*

Among central European *Agabus* species, *A. uliginosus* is characterized by metacoxal lines fully reaching the hind margin of the metasternum and broad metasternal wings, according to Schaeflein (1971) and Nilsson and Holmen (1995). Additionally, the anterior pronotal puncture lines are continuous and not distinctly interrupted. The meshes of elytral sculpture are typically small, smaller than elytral punctures, as noted by Ganglbauer (1892). Characteristically, it exhibits a strongly convex dorsal surface and very broad pronotal lateral margins. Nilsson and Petrov (2006) separated *A. uralensis* from *A. uliginosus*, as a vicariant species in the western Palaearctic, based on its smaller body size and shorter, evenly tapering penis. As several authors before, recently Turner et al. (2015) noted high variance in several external morphological characteristics of *A. uliginosus*, including microsculpture and coloration. Stephens (1828) described stronger metallic specimens from England, while Queney (2002) observed a rufous form from France. Moreover, *A. uliginosus*, uniquely in the subfamily Agabinae, shows inter- and intrasexual dimorphism within part of its distribution area (Bilton et al. 2016).

Biology and population structure of *A. uliginosus*

The life cycle of *A. uliginosus* is characterized as an univoltine spring breeder with summer larvae and overwintering adults (Nilsson 1986). Galewski (1968, 1980) observed second-stage larvae from early May to late July in Poland, followed by third-stage larvae from late April to mid-September. He noted the presence of mostly third-stage larvae but also pupae and immature adults during May, suggesting a prolonged oviposition period. Nilsson (1986) suggested the possibility of an extended oviposition period as well. Similar bionomical data were reported by Balfour-Browne (1950). In contrast, Braasch (1989) proposed that *A. uliginosus* has two oviposition periods: the first from March to April and the second from July to August in Northeastern Germany. He demonstrated two peaks of adult beetles, one from January to March after adult hibernation and another from the end of May to October. Two peaks were also seen in both second- and third-stage larvae, with the first peak from April to the beginning of May and the second peak in July. Braasch (1989) also noted that *A. uliginosus* larvae are typically found in temporary waters, whereas adults mostly inhabit permanent water bodies and migrate between the two types of habitats at least for oviposition. Hendrich (2003) reported an interesting phenomenon: “After its habitats dry out in May and June, and shortly after hatching of the new generation, the species undertakes longer land migrations in large numbers, sometimes taking them more than 20 meters from the water. In the years 1985–1989, Ralph Platen was able to detect large numbers of *A. uliginosus* in various fen areas in Berlin using pitfall traps. Of the more than 1,500 diving beetles captured as part of a research project, more than 800 specimens were *A. uliginosus*”. Spitzenberg (2021) reported a phenological maximum for *A. uliginosus* during May in Saxony-Anhalt and observed avoidance of higher elevations. He also described the ecological preferences of the species for eutrophic ponds, backwaters,

ditches, canals, salt lakes, and salt marshes in this area. Additionally, Spitzenberg (2021) reported flight activity at light, a behavior supported by Kehl and Dettner (2007), who associated this species with category 2a, indicating the presence or absence of flight muscles depending on individuals. Considering the above facts, that the larvae develop in seasonal waters, and migration is a significant investment for the adults, it is possible that the resulting time stress is the reason they evolved this special kind of genital development. Although it is only an assumption, it is likely to be beneficial to minimize the time of larval and pupal development, and therefore building up as little body biomass as possible in these periods, leaving some of the development to be enabled by the subsequent feeding of the adults taking place in more permanent waters.

Summarizing available records from Central Europe, we see a peak in records during March-April-May for both species, consistent with the phenological observations mentioned above. Additionally, the exclusive presence of specific records of *A. lotti* during these months supports the hypothesis that this species may actually represent young individuals of *A. uliginosus* returning to the water shortly after hatching, potentially contributing to the surge in density, and consequently in number of records. However, it is essential to consider that climatic variations and differences in altitude make objective phenological comparisons challenging. For instance, March marks the beginning of the breeding season in southern and lowland areas, whereas reproduction may commence later, typically in May, in higher elevations. Additionally, data on A. 'lotti' have been documented in mountainous regions of Italy, Austria, and Slovakia.

As demonstrated by Classen and Dettner (1983) and Dettner et al. (1986), age structures within dytiscid populations can exhibit significant fluctuations from month to month, during the course of one or several years, although the exact lifespan of many dytiscid species, including *A. uliginosus*, remains unclear. In species such as *Agabus bipustulatus* (Linnaeus, 1767), *A. paludosus* (Fabricius, 1801), or *Platambus maculatus* (Linnaeus, 1758), populations typically consist of varying numbers of individuals across different age classes at any given time, and these proportions are constantly changing. These age classes are delineated based on the developmental status of internal gonads, which can only be reliably assessed in freshly killed or recently ethanol-preserved specimens. Therefore, any additional records or notes accompanying regular distribution data regarding the first occurrences of larval stages or immature (soft, unsclerotized) adults or information on the status of the gonads provide crucial information for determining the life-cycle type of each species, in accordance with the findings of Nilsson (1986).

Age dependency of male sexual organs of *A. uliginosus*

Within the *uliginosus* group, and likely also in the *punctulatus* group, Nilsson and Petrov (2006) suggested that during the imaginal stage, the degree of sclerotization and enlargement of the phallobase of the aedeagus might increase. After studying larger material of *A. uliginosus* and *A. uralensis*, they concluded that identification might be problematic if the phallobase did not enlarge since the development of the basal apodeme occurs later than that of the rest of the penis. This is unusual because sclerotized aedeagi of male insects typically represent morphologically stable, constant, and highly useful diagnostic

structures, allowing species differentiation in most insect groups. The lock-and-key mechanism (Eberhard 1985) posits that the morphologically species-specific external genitalia of elder specimens correspond tightly between both sexes. However, the aedeagal morphology of freshly hatched male beetles has not been systematically analyzed before and has not been compared with appropriate structures of elder specimens, including the *A. uliginosus* group.

As reported by Classen and Dettner (1983) in *A. bipustulatus* and *A. paludosus*, it was expected, and now demonstrated for the first time in *A. uliginosus* s. l. (Fig. 1H–J), that the lengths and diameters of ectadenies gradually increase from one age class to another. Additionally, and in line with Dettner et al. (1986), the fresh weights also positively correlated with the lengths of "b+c" (Fig. 2A, see also Fig. 1A). At the same time, we clearly showed that aedeagal morphology gradually changes with age: the phallobase enlarges, its basal part becomes increasingly sclerotized, the parameres and tegmina lengthen from immature, young specimens (see Figs 1H, 3A–C, 4A, B) to elder and highly aged adults (Figs 1I–J, 3D, 4C, D). During this process, younger adults resemble *A. lotti* more, while elder and fully sclerotized adults resemble *A. uliginosus* s. str. more. Within Dytiscidae, beside the *Agabus uliginosus* group, Nilsson and Petrov (2006) mentioned a comparable case in *Hygrotus tumidiventris* (Fall, 1919), with a normal aedeagus figured by Larson (1975).

While they are not explicitly sexual organs, the foreclaws of males also play a role in the mating process, thus becoming worn out as specimens age, with their apex becoming blunted, deformed, and eventually breaking off. We hypothesize that the fixing and adhering of male foreclaws to the pronoti of females during one or more extended copulations, particularly with increasing age, may damage both the thin and sensitive arched apodemes situated at the base as well as the tips of the foreclaws.

Our results also underscore the urgent need for comparisons of aedeagal morphologies of young teneral males with those of adult specimens, not only in Dytiscidae but also in other taxa. This is particularly crucial for older descriptions where figures of aedeagi were provided in dorsal views. When large amounts of viscous ectadenial materials, especially proteins and mucosubstances, as seen in Carabidae (see Krüger et al. 2014; Schubert et al. 2017), pass through aedeagi, these structures, if not completely sclerotized, may swell enormously. Depending on the amounts and consistency of ectadenial secretions, a certain flexibility of aedeagal structures is essential.

Molecular diversity and MOTU delimitation

DNA barcoding is already a well-established and powerful tool for molecular identification of the species (Grant et al. 2021; Antil et al. 2023). Beetles are among the flagship groups of invertebrates for which barcode reference libraries are built (Pentinsaari et al. 2014; Hendrich et al. 2015). Still, even in Europe, progress reached above 50% of known species for freshwater aquatic beetles (Csabai et al. 2023). Species delimitation methods revealed two (ASAP), three (BIN), and four (mPTP) MOTUs; these also considered morphological features, and the accepted approach can be considered as a rough proxy of species. We should consider the method limitations and interpretation of the obtained result. The conservative approach of the ASAP method seems congruent with

our morphological investigation (Copilaş-Ciocianu et al. 2022), and the mPTP approach tended to over-split (Song et al. 2018; Goulpeau et al. 2022). While the phenomenon of bin-sharing may arise here (e.g., Raupach et al. 2018, 2020; Schattanek-Wiesmair et al. 2024), we can rule out this possibility since in some cases, the sequences are 100% identical for individuals classified morphologically as different types. This holds even when comparing Hungarian *A. lotti* individuals with German and Finnish *A. uliginosus* s. str. As a theoretical possibility, it could be argued that all previous COI sequences in BOLD may have originated from *A. lotti*. However, this can be clearly ruled out based on the attached photos of processed individuals in BOLD, especially with reference to the specimen with Process-ID COLFE1294-13, where the genitals (aedeagus and parameres) are visible, leaving no doubt that this old specimen belongs to *A. uliginosus* s. str.

The addition of the Hungarian sequences significantly increased the known haplotype diversity within the species. Large number of individuals are available from Germany, Finland, and Hungary in BOLD, and the Hungarian dataset stands out as the most heterogeneous. Only available molecular data from *A. lotti* are deposited in Venables' thesis (2016). One specimen from Italy is closely related to *A. uliginosus* from Germany. However, it is noteworthy that Venables utilized COI-3P sequences, and unfortunately, the sequence corresponding to *A. lotti* is not publicly accessible in repositories. Despite this limitation, the observed genetic divergence appears relatively modest. Given the geographical context (Italy), it is plausible to consider the possibility of intraspecific variation induced by spatial isolation, although definitive conclusions warrant further investigation.

The comparison of sequences confirmed with high confidence that individuals classified as *A. lotti* and *A. uliginosus* belong to the same species based on COI markers. Although it nicely supports our single-species hypothesis in the present case, the COI analysis alone is insufficient for a comprehensive revision of the species complex. It would be highly beneficial to conduct a comprehensive multi-marker study encompassing most of the distribution range of the species group, covering various regions of the Holarctic region, including all known species (currently six without *A. lotti*), and employing integrated molecular and morphological approaches, including analysis of immature, young, and teneral individuals. Limited sampling size may also affect our findings.

The case of the #H27 specimen

As shown, specimen #H27 (morphologically identified as *A. uliginosus* s. str.) displayed molecular deviations from all other specimens, irrespective of their morphological identification as *A. uliginosus* s. str. or *A. lotti*. Therefore, detailed comparisons were made of the aedeagi, parameres, tegmina, male foreclaws, and internal gonads of the molecularly analyzed specimens. Young specimens were characterized by non-sclerotized, slightly colored, translucent genital sclerites, small and lengthened tegmina, as well as thin and small bases of parameres (Fig. 4A right; #H19). In contrast, elder specimens exhibited thickened and more sclerotized tegmina, with thickened and sclerotized bases of parameres (Fig. 4B right; #H11). Specimen #H27 (Fig. 4C right) was notably older compared to #H19 (Fig. 4A right), displaying typical features of *A. uliginosus* s. str., including an increased phallobase and parameres. However, #H27 had a lengthened teg-

men that was only partially sclerotized in the center and not at the base. Exhibiting non-sclerotized internal organs, #H27 (c+d = 0.42 mm; d.ej.: 0.18 mm; ect.: 3.5 mm) appeared slightly younger than #H11 (c+d = 0.34 mm; d.ej.: 0.25 mm; ect.: 5.1 mm, see also Suppl. material 1: table S1). Nevertheless, based on its sclerotized and broader phallobase, #H27 was conclusively older than #H11. Age determination was further supported by the exclusive presence of sperms in the spermatic ducts (sd) of #H27 (Fig. 3E), contrasting with their absence in #H11 and #H19. Although specimens from the Hungarian population were not very old, external genitalia were compared to distinctly elder *A. uliginosus* specimens from other regions (despite the unavailability of internal genitalia). Two specimens (#G01, #G02) from Salemer Moor/Ratzeburg, Germany, both with c+d values of 0.4 mm, large phallobases, and thick and sclerotized basal pieces of parameres, showed characteristic lengthened tegmina similar to those seen in #H27. This comparison further confirmed that Hungarian specimen #H27 represented an elder specimen of *A. uliginosus* compared to #H11. Furthermore, the larger asymmetrical foreclaw was compared among these three Hungarian and one German male specimens (Fig. 4A–D left). The youngest specimen, #H19, displayed a highly arched basal apodeme with a very sharp tip (Fig. 4A left). In the somewhat elder specimen #H11, the basal apodeme of the sharp claw (only one leg was available) appeared slightly slender and frayed out (Fig. 4B left). Specimen #H27 was characterized by a worn-out basal apodeme and rounded or broken tips of the claws (Fig. 4C left), indicating that #H27 is the eldest specimen among the three Hungarian specimens depicted. The old specimen from Germany exhibited a male foreclaw with a rounded tip and a slightly frayed basal apodeme (Fig. 4D left). The case of specimen #27 unequivocally indicates that while current knowledge and examination of northern populations have revealed a relative genetic homogeneity, parts of the distribution area not yet genetically characterized, particularly the southern and eastern areas, may harbor unexpected discoveries, potentially exhibiting a broader diversity of haplotypes and even revealing previously unknown hidden species. As shown by Bergsten et al. (2012), in a comprehensive study 250 individuals were needed to demonstrate 95% of the molecular variation of the widespread species *Agabus bipustulatus*, and approximately 70 for other species with more restricted distributions. More extensive sampling in the Pannonian Basin may fill the gaps regarding *A. uliginosus* group, as this region is proven to be a biodiversity hotspot for several aquatic invertebrate groups (Csapó et al. 2020; Sworobowicz et al. 2020). With limited sampling, we cannot exclude or confirm the phylogeographic effect of molecular separation of specimen #27. Multiple BINs within species are not unique (Raupach et al. 2020; Geiger et al. 2021).

Conclusions and the taxonomic position of *Agabus lotti*

All our findings indicate that beetles identified as *Agabus lotti* represent young specimens of *A. uliginosus*. Therefore, *Agabus lotti* Turner, Toledo & Mazzoldi, 2015 must be regarded as a junior synonym (syn. nov.) of *Agabus uliginosus* (Linnaeus, 1761). We observed and demonstrated a gradual morphological transition of aedeagal features from young specimens (*A. lotti*) to elder individuals (*A. uliginosus*), corresponding with age-dependent variations of internal sexual organs. Obviously, Turner et al. (2015) was unable to analyze both sclerotized and soft

structures across a series of male specimens of different ages. However, they noted in their description of *A. lotti* that the endophallus varies morphologically depending on the maturity of the beetles and referenced remarks by Nilsson and Petrov (2006), who observed enlarged aedeagi in mature *A. uralensis* specimens and underdeveloped ones in young teneral specimens. Additionally, the fact that nearly all localities of *A. lotti* are found within the geographic range of *A. uliginosus* (with exceptions possibly in Central Italy) further challenges the species priority of *A. lotti*. Our molecular analyses further reinforced our hypothesis that *A. lotti* and *A. uliginosus* are the same species, as we observed only minimal differences among most specimens, with all but one individual classified into a BIN demonstrating high homogeneity. However, the genetically distinct nature of a single individual also underscores the need for further investigations in the species group. Particularly, with regards to the possible existing but never analyzed southern populations, more significant differences may exist, or the possibility of discovering new, previously unknown species cannot be ruled out.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

KD and ZC conceived the ideas and designed methodology; ZK and ZC conducted the fieldwork; KD, ZK, and TR conducted various morphological and molecular laboratory work to collect the data; ZC, KD and TR analyzed the data; KD and ZC led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. Our study brings together authors from a number of different countries, including scientists based in the countries where the study was conducted. All authors were engaged with the research and study design to ensure that the diverse sets of perspectives they represent were considered. Where relevant, literature published by scientists from the region was cited.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215: 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Antil S, Abraham JS, Sripoorna S, Maurya S, Dagar J, Makhija S, Bhagat P, Gupta R, Sood U, Lal R, Toteja R (2023) DNA barcoding, an effective tool for species identification: a review. Molecular Biology Reports 50: 761–775. https://doi.org/10.1007/s11033-022-08015-7

Astrin JJ, Stüben PE (2008) Phylogeny in cryptic weevils: molecules, morphology and new genera of western Palaearctic Cryptorhynchinae (Coleoptera: Curculionidae). Invertebrate Systematics 22: 503–522. https://doi.org/10.1071/IS07057

Balfour-Browne F (1950) British Water Beetles. Vol. II, Ray Society, London.

Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2013) GenBank. Nucleic Acids Research 41(D1): D36–D42. https://doi.org/10.1093/nar/gks1195

Bergsten J, Bilton DT, Fujisawa T, Elliott M, Monaghan MT, Balke M, Hendrich L, Geijer J, Herrmann J, Foster GN, Ribera I, Nilsson AN, Barraclough TG, Vogler AP (2012) The effect of geographical scale of sampling on DNA barcoding. Systematic Biology 61(5): 851–869. https://doi.org/10.1093/sysbio/sys037

Bilton DT, Hayward JWG, Rocha J, Foster GN (2016) Sexual dimorphism and sexual conflict in the diving beetle *Agabus uliginosus* (L.) (Coleoptera: Dytiscidae). Biological Journal of the Linnean Society 119: 1089–1095. https://doi.org/10.1111/bij.12850

Blunck H (1912) Das Geschlechtsleben des *Dytiscus marginalis* L. Die Begattung. Zeitschrift für wissenschaftliche Zoologie 102: 169–248.

Boda P, Móra A, Csabai Z (2019) Aquatic macroinvertebrates from soda pans and adjacent wetland habitats of the Hungarian Puszta region with first records of four species from Hungary. Spixiana 42: 263–282.

Boda P, Móra A, Csabai Z (2023) AMI-KMNP dataset: Occurrence records of aquatic macroinvertebrate species from a 10-year-long biodiversity survey in SE Hungary. Journal of Limnology 82(S1): 2118. https://doi.org/10.4081/jlimnol.2023.2118

Braasch D (1989) *Agabus uliginosus* (L., 1761), eine bivoltine Art? Entomologische Nachrichten und Berichte 33: 91–93.

Casquet J, Thebaud C, Gillespie RG (2012) Chelex without boiling, a rapid and easy technique to obtain stable amplifiable DNA from small amounts of ethanol-stored spiders. Molecular Ecology Resources 12: 136–141. https://doi.org/10.1111/j.1755-0998.2011.03073.x

Classen R, Dettner K (1983) Pygidial defensive titer and population structure of *Agabus bipustulatus* L. and *Agabus paludosus* F. (Coleoptera, Dytiscidae). Journal of Chemical Ecology 9: 201–209. https://doi.org/10.1007/BF00988037

Copilaş-Ciocianu D, Rewicz T, Sands AF, Palatov D, Marin I, Arbačiauskas K, Hebert PDN, Grabowski M, Audzijonyte A (2022) A DNA barcode reference library for endemic Ponto-Caspian amphipods. Scientific Reports 12: 11332. https://doi.org/10.1038/s41598-022-15442-w

Csabai Z, Čiamporová-Zaťovičová Z, Boda P, Čiampor F (2023) 50%, not great, not terrible - Pan-European gap-analysis shows the real status of the DNA barcode reference libraries in two aquatic invertebrate groups and points the way ahead. Science of the Total Environment 863: 160922. https://doi.org/10.1016/j.scitotenv.2022.160922

Csapó H, Krzywoźniak P, Grabowski M, Wattier R, Bącela-Spychalska K, Mamos T, Jelić M Rewicz T (2020) Successful post-glacial colonization of Europe by single lineage of freshwater amphipod from its Pannonian Plio-Pleistocene diversification hotspot. Scientific Reports 10(1): 18695. https://doi.org/10.1038/s41598-020-75568-7

Dettner K, Hübner M, Classen R (1986) Age structure, phenology and prey of some reophilic Dytiscidae (Coleoptera). Entomologica Basiliensia 11: 343–370.

Eberhard WG (1985) Sexual Selection and Animal Genitalia. MA: Harvard University Press, Cambridge. https://doi.org/10.4159/harvard.9780674330702

Fery H, Bouzid S (2016) Notes on *Graptodytes* Seidlitz, 1887, re-instatement of *G. laeticulus* (Sharp, 1882) as valid species and description of *Tassilodytes* nov.gen. from Algeria (Coleoptera, Dytiscidae, Hydroporinae, Siettitiina). Linzer Biologische Beiträge 48(1): 451–481.

Franciscolo ME (1979) Coleoptera Haliplidae. Hygrobiidae, Gyrinidae, Dytiscidae. Fauna d'Italia XIV. Calderini, Bologna.

Galewski K (1968) Descriptions of larvae of *Agabus uliginosus* (L.) and *A. congener* (Thunb.) (Coleoptera, Dytiscidae). Annales Zoologici 26: 323–332.

Galewski K (1980) Third stage larvae of European species of *Agabus* Leach (Coleoptera, Dytiscidae). Polski Pismo Entomologiczne 50: 3–69.

Ganglbauer L (1892) Die Käfer von Mitteleuropa. 1. Bd. Familienreihe Caraboidea. C. Gerold`s Sohn, Wien.

Geiger M, Koblmüller S, Assandri G, Chovanec A, Ekrem T, Fischer I, Galimberti A, Grabowski M, Haring E, Hausmann A, Hendrich L, Koch S, Mamos T, Rothe U, Rulik B, Rewicz T, Sittenthaler M, Stur E, Tończyk G, Zangl L, Moriniere J (2021) Coverage and quality of DNA barcode references for Central and Northern European Odonata. PeerJ 9: e11192. https://doi.org/10.7717/peerj.11192

Goulpeau A, Penel B, Maggia ME, Marchán DF, Steinke D, Hedde M, Decaëns T (2022) OTU delimitation with earthworm DNA barcodes: A comparison of methods. Diversity 14(10): 866. https://doi.org/10.3390/d14100866

Grant DM, Brodnicke OB, Evankow AM, Ferreira AO, Fontes JT, Hansen AK, Jensen MR, Kalaycı TE, Leeper A, Patil SK, Reunamo A, Roberts AJ, Shigdel R, Tyukosova V, Brendiksby M, Blaalid R, Costa FO, Hollingsworth PM, Stur E, Ekrem T (2021) The Future of DNA Barcoding: Reflections from Early Career Researchers. Diversity 13: 313. https://doi.org/10.3390/d13070313

Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends in Genetics 23(4): 167–172. https://doi.org/10.1016/j.tig.2007.02.001

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. Proceedings of the Royal Society London B 270: 313–321. https://doi.org/10.1098/rspb.2002.2218

Hebert PDN, Ratnasingham S, deWaard JR (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society London B 270: S96–S99. https://doi.org/10.1098/rsbl.2003.0025

Hendrich L (2003) Die Wasserkäfer von Berlin. Struktur der aquatischen Käferfauna (Hydradephaga, Hydrophiloidea, Dryopoidea (partim) and Staphylinoidea (partim) in anthropogen beeinflussten Gewässern von Berlin – Taxonomische, räumliche, faunistische und ökologische Aspekte. PHD Thesis, Technische Universität, Berlin.

Hendrich L, Morinière J, Haszprunar G, Hebert PDN, Hausmann A, Köhler F, Balke M (2015) A comprehensive DNA barcode database for Central European beetles with a focus on Germany: adding more than 3500 identified species to BOLD. Molecular Ecology Resources 15: 795–818. https://doi.org/10.1111/1755-0998.12354

Hou Z, Fu J, Li S (2007) A molecular phylogeny of the genus *Gammarus* (Crustacea: Amphipoda) based on mitochondrial and nuclear gene sequences. Molecular Phylogenetics and Evolution 45: 596–611. https://doi.org/10.1016/j.ympev.2007.06.006

Kapli P, Lutteropp S, Zhang J, Kobert K, Pavlidis P, Stamatakis A, Flouri T (2017) Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. Bioinformatics 33(11): 1630–1638. https://doi.org/10.1093/bioinformatics/btx025

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Kehl S, Dettner K (2007) Flugfähigkeit der in Deutschland vorkommenden adephagen Wasserkäfer (Coleoptera, Hydradephaga). Entomologie heute 19: 141–161.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111–120. https://doi.org/10.1007/BF01731581

Krüger S, Ferenz HJ, Randall M, Hodgson AN (2014) Structure of the male reproductive accessory glands of *Pterostichus nigrita* (Coleoptera: Carabidae), their role in spermatophore formation. Invertebrate Reproduction and Development 58(2): 75–88. https://doi.org/10.1080/07924259.2013.822835

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35: 1547–1549. https://doi.org/10.1093/molbev/msy096

Larson DJ (1975) Dytiscidae of Alberta. Quaestiones Entomologicae 11: 243–498.

Larson DJ (1989) Revision of North American *Agabus* Leach (Coleoptera: Dytiscidae): introduction, key to species groups, and classification of the *ambiguus*-, *tristis*-, and *arcticus*-groups. The Canadian Entomologist 121: 861–919. https://doi.org/10.4039/Ent121861-10

Larson DJ, Alarie Y, Roughley RE (2000) Predaceous diving beetles (Coleoptera: Dytiscidae) of the nearctic region, with emphasis on the fauna of Canada and Alaska. NRC Research Press, Ottawa, ON, Canada.

Lawrence JF, Beutel RG, Leschen RAB, Slipinski A (2010) 2. Glossary of Morphological Terms. In: Leschen RAB, Beutel, RG, Lawrence JF (Eds) Coleoptera, Beetles, Morphology and Systematics, Vol. 2. De Gruyter, Berlin, New York, 9–20. https://doi.org/10.1515/9783110911213.9

Leigh JW, Bryant D (2015) POPART: full-feature software for haplotype network construction. Methods in Ecology and Evolution 6: 1110–1116. https://doi.org/10.1111/2041-210X.12410

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Linard B, Crampton-Platt A, Moriniere J, Timmermans MJ, Andujar C, Arribas P, Miller KE, Lipecki J, Favreau E, Hunter A, Gómez-Rodríguez C, Barton C, Nie R, Gillett CPDT, Breeschoten T, Bocak L, Vogler, AP (2018) The contribution of mitochondrial metagenomics to large-scale data mining and phylogenetic analysis of Coleoptera. Molecular Phylogenetics and Evolution 128: 1–11. https://doi.org/10.1016/j.ympev.2018.07.008

Miller KB, Bergsten J (2016) Diving Beetles of the World. Johns Hopkins University Press, Baltimore, MD.

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York. https://doi.org/10.7312/nei-92038

Nilsson AN (1986) Life cycles and habitats of the Northern European Agabini (Coleoptera, Dytiscidae). Entomologica Basiliensia 11: 391–417.

Nilsson AN (2000) A new view on the generic classification of the *Agabus*-group of genera of the Agabini, aimed at solving the problem with a paraphyletic *Agabus* (Coleoptera: Dytiscidae). Koleopterologische Rundschau 70: 17–36.

Nilsson AN, Hájek J (2024) A World Catalogue of the Family Dytiscidae. Version 1.I.2024 [a corrected and updated version for the printed cataloge: Nilsson AN 2001: Dytiscidae (Coleoptera). World Catalogue of Insects. Vol. 3. Stenstrup: Apollo Books, 395 pp.]. https://waterbeetles.eu/documents/W_CAT_Dytiscidae_2024_new.pdf [Accessed on 02.02.2024]

Nilsson AN, Holmen M (1995) The aquatic Adephaga (Coleoptera) of Dennoscandia and Denmark. II. Dytiscidae. Fauna Entomologica Scandinavica 32. E. J. Brill, Leiden. https://doi.org/10.1163/9789004273603

Nilsson AN, Petrov PN (2006) On the identity of *Agabus uliginosus* (Linnaeus), with the description of a new species of *Agabus* from Russia (Coleoptera: Dytiscidae). Russian Entomological Journal 14(3) (2005): 1–9.

Nilsson AN, Toledo M (1999) *Agabus amnicola* (J. Sahlberg) and *A. jacobsoni* Zaitzev, two poorly known species of the *uliginosus*-group (Coleoptera: Dytiscidae). Koleopterologische Rundschau 69: 29–32.

Pentinsaari M, Hebert PDN, Mutanen M (2014) Barcoding Beetles: A regional survey of 1872 species reveals high identification success and unusually deep interspecific divergences. PLOS ONE 9(9): e108651. https://doi.org/10.1371/journal.pone.0108651

Puillandre N, Brouillet S, Achaz G (2021) ASAP: assemble species by automatic partitioning. Molecular Ecology Resources 21: 609–620. https://doi.org/10.1111/1755-0998.13281

Queney P (2002) Voyage avec les *Agabus biguttatus* (Olivier) et *uliginosus* (Linné) et quelques autres congénères (Coleoptera: Dytiscidae). Le Coléoptériste 5(3): 155–159.

Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes 7: 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Ratnasingham S, Hebert PDN (2013) A DNA-based registry for all animal species: The Barcode Index Number (BIN) System. PLOS ONE 8(7): e66213. https://doi.org/10.1371/journal.pone.0066213

Raupach MJ, Hannig K, Morinière J, Hendrich L (2018) A DNA barcode library for ground beetles of Germany: the genus *Amara* Bonelli, 1810 (Insecta, Coleoptera, Carabidae). ZooKeys 759: 57–80. https://doi.org/10.3897/zookeys.759.24129

Raupach MJ, Hannig K, Morinière J, Hendrich L (2020) A DNA barcode library for ground beetles of Germany: the genus *Pterostichus* Bonelli, 1810 and allied taxa (Insecta, Coleoptera, Carabidae). ZooKeys 980: 93–117. https://doi.org/10.3897/zookeys.980.55979

Rocci S, Terzani F, Mascagni A (2021) Coleotterofauna acquatica e semiacquatica dell'Umbria (Insecta: Coleoptera: Gyrinidae, Haliplidae, Noteridae, Hygrobiidae, Dytiscidae, Helophoridae, Georissidae, Hydrochidae, Hydrophilidae, Hydraenidae, Scirtidae, Elmidae, Dryopidae, Limnichidae, Heteroceridae, Psephenidae, Chrysomelidae, Curculionidae). Quaderno di Studi e Notizie di Storia Naturale della Romagna 53: 65–153. https://doi.org/10.4081/bollettinosei.2021.3

Rulik B, Eberle J, von der Mark L, Thormann J, Jung M, Köhler F, Apfel W, Weigel A, Kopetz A, Köhler J, Fritzlar F, Hartmann M, Hadulla K, Schmidt J, Hörren T, Krebs D, Theves F, Eulitz U, Skale A, Rohwedder D, Kleeberg A, Astrin JJ, Geiger MF, Wägele JW, Grobe P, Ahrens D (2017) Using taxonomic consistency with semi-automated data pre-processing for high quality DNA barcodes. Methods Ecology and Evolution 8: 1878–1887. https://doi.org/10.1111/2041-210X.12824

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Schaeflein H (1971) 4. Familie: Dytiscidae, echte Schwimmkäfer. In: Freude H, Harde KW, Lohse GA (Eds) Die Käfer Mitteleuropas, Bd. 3. Krefeld: Goecke & Evers, 16–90.

Schattanek-Wiesmair B, Huemer P, Wieser C, Stark W, Hausmann A, Koblmüller S, Sefc KM (2024) A DNA barcode library of Austrian geometridae (Lepidoptera) reveals high potential for DNA-based species identification. PLOS ONE 19(3): e0298025. https://doi.org/10.1371/journal.pone.0298025

Schubert LF, Krüger S, Moritz GB, Schubert V (2017) Male reproductive system and spermatogenesis of *Limodromus assimilis* (Paykull 1790). PLOS ONE 12(7): e0180492. https://doi.org/10.1371/journal.pone.0180492

Seifert G (1995) Entomologisches Praktikum. 3., neubearbeitete und erweiterte Auflage. Georg Thieme Verlag, Stuttgart.

Song C, Lin XL, Wang Q, Wang XH (2018) DNA barcodes successfully delimit morphospecies in a superdiverse insect genus. Zoologica Scripta 47(3): 311–324. https://doi.org/10.1111/zsc.12284

Spitzenberg D (2021) Die wasserbewohnenden Käfer Sachsen-Anhalts. Rangsdorf: Natur & Text.

Stephens JF (1828) Illustrations of British entomology. Mandibulata. Vol. 2. Baldwin & Cradock, London. https://doi.org/10.5962/bhl.title.50040

Sworobowicz L, Mamos T, Grabowski M, Wysocka A (2020) Lasting through the ice age: The role of the proglacial refugia in the maintenance of genetic diversity, population growth, and high dispersal rate in a widespread freshwater crustacean. Freshwater Biology 65(6): 1028–1046. https://doi.org/10.1111/fwb.13487

Turner CR, Toledo M, Mazzoldi P (2015) A previously unrecognized species of *Agabus* Leach, 1817 in Italy and Central Europe. Koleopterologische Rundschau 85: 1–6.

Venables C (2016) The molecular systematics of the Agabinae Thomson, 1867 (Coleoptera, Dytiscidae). Doctoral Thesis, University of Calgary. https://doi.org/10.11575/PRISM/28250

Vences M, Miralles A, Brouillet S, Ducasse J, Fedosov A, Kharchev V, Kumari S, Patmanidis S, Puillandre N, Scherz MD, Kostadinov I, Renner SS (2021) iTaxoTools 0.1: Kickstarting a specimen-based software toolkit for taxonomists. Megataxa 6: 77–92. https://doi.org/10.11646/megataxa.6.2.1

Weigand H, Beermann AJ, Čiampor F, Costa FO, Csabai Z, Duarte S, Geiger MF, Grabowski M, Rimet F, Rulik B, Strand M, Szucsich N, Weigand AM, Willassen E, Wyler SA, Bouchez A, Borja A, Čiamporová-Zaťovičová Z, Ferreira S, Dijkstra KD, Eisendle U, Freyhof J, Gadawski P, Graf W, Haegerbaeumer A, van der Hoorn BB, Japoshvili B, Keresztes L, Keskin E, Leese F, Macher JN, Mamos T, Paz G, Pešić V, Pfannkuchen D M, Pfannkuchen MA, Price BW, Rinkevich B, Teixeira MAL, Várbíró G, Ekrem T (2019) DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. Science of the Total Environment 678: 499–524. https://doi.org/10.1016/j.scitotenv.2019.04.247

Supplementary material 1

Five supplementary tables

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Data type: xlsx

Explanation note: Five tables supplementing the submitted manuscript. They contain all raw data and calculated data which are not available elsewhere. Morphological measurements, occurrence data, database citations, BOLD specimen data, genetic distances.

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